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Hepatoactive substances eliminated by continuous venovenous hemofiltration in acute renal failure patients

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Background. Acute renal failure (ARF) in critically ill patients is mostly part of a multi-organ failure. Therefore, the effects of renal replacement therapy on the liver are clinically important. We investigated the effects of ultrafiltrates of patients treated with continuous venovenous hemofiltration (CVVH) on liver cells *in vitro.*

Methods. Patients with ARF were consecutively treated with CVVH using Multiflow60 (group I) or FH66 filters (group II). They were comparable with respect to diagnosis, age, sex, laboratory parameters, and renal replacement treatment, but were different in daily diuresis, serum levels, and blood flow. Ultrafiltrates were collected within the first 10 minutes after change of hemofilter. Proliferation (bromodeoxyuridine), vitality (lactate dehydrogenase), and acute-phase protein secretion of HepG2 cells were measured.

Results. Ultrafiltrates changed liver cell function significantly compared with medium control. Proliferation (group I 29.8 ± 5.2% vs. group II 48.4 ± 6.6%, $P < 0.05$) and vitality (group I 78.7 ± 2.0% vs. group II 87.6 ± 1.7%, $P < 0.01$) of HepG2 cells were significantly different. On the one hand, the secretion of the negative acute-phase protein transferrin [group I 3.1 ± 0.2 (ng/μg protein) vs. group II 5.1 ± 0.5 (ng/μg protein), $P < 0.01$] was significantly reduced by Multiflow60 ultrafiltrates. On the other hand, positive acute-phase protein α1-acid glycoprotein was significantly stimulated by Multiflow60 ultrafiltrates [group I 2.6 ± 0.1 (ng/μg protein) vs. group II 1.7 ± 0.1 (ng/μg protein), $P < 0.001$].

Conclusion. This study demonstrates hepatoactive mediators in the ultrafiltrates. They are hepatotoxic and influence acute-phase protein metabolism. Further studies have to elucidate the different effects in both groups and the analysis of the putative mediator(s). It remains a challenging task to consider therapeutic measures to optimize renal replacement therapy in critically ill patients.

Acute renal failure (ARF) in the intensive care unit (ICU) is a multifactorial clinical syndrome that is characterized by an abrupt but potentially reversible reduction in excretory renal function. The prognosis of patients with ARF has not improved during the past decades despite modern critical care medicine. On the one hand, this fact could be explained by the rising number of patients with multiple organ failure (MOF). On the other hand, ARF in the critical care setting is rarely a single organ failure, but mostly occurs as part of a MOF [1]. In patients with MOF, mortality directly correlates with the number of failing organ systems [1]. An additional hepatic failure was found to be especially and significantly related to mortality [2]. Continuous forms of renal replacement therapies (CRRTs) with a polyacrylonitrile membrane were shown to improve the clinical course and to increase the survival of patients with hepatic failure and ARF [3]. Hemofiltration (HF) using polyacrylonitrile membrane resulted in smaller adverse changes in intracranial pressure, mean arterial blood pressure, cerebral perfusion pressure, and tissue oxygen delivery [4]. The increase in survival could be due to the removal of middle molecular weight compounds, which have been thought to be important in the pathogenesis of hepatic coma.

Correspondingly, toxic mediators (cytokines, cyclic endoperoxides, and so forth) have been identified in ultrafiltrates taken during continuous venovenous HF (CVVH) with polyamide from septic patients [5]. However, the possibility that HF also improves specific organ failure in MOF is a point of controversy.

The aim of this study was to examine hepatoactive mediators in the ultrafiltrate of critically ill patients with ARF.

Therefore, we investigated the effects of ultrafiltrates of patients treated either with Multiflow60 or FH66 on liver cells *in vitro* (HepG2). Several parameters of cell function [proliferation, lactate dehydrogenase (LDH)-release, acute-phase protein secretion] were measured.

METHODS

Patients

Thirty-one patients with ARF at the ICU of the University Hospital of Saarland were studied. ARF was considered when urine output was <200 ml/12 hr, despite maximal conventional therapy, and at least one addi-

Key words: multiple organ failure, critical care, ultrafiltrates, hepatotoxins, continuous venovenous hemofiltration.

Table 1. Patient characterization

	Group I ($N = 16$)	Group II ($N = 15$)	P value
Gender *female/male*	7/9	7/8	
Age *years*	60.3 ± 4.6	56.1 ± 4.9	NS
Serum creatinine *mg/dl*	3.0 ± 0.3	3.2 ± 0.3	NS
Urea *mg/dl*	117.8 ± 13.7	163.6 ± 17.0	<0.05
Uric acid *mg/dl*	13.1 ± 7.9	7.2 ± 1.5	NS
Residual rest function *ml/24 hr*	1085.3 ± 313.7	341.0 ± 147.4	<0.05
GOT *U/liter*	181.8 ± 81.2	52.9 ± 18.9	NS
GPT *U/liter*	79.2 ± 43.6	53.7 ± 26.8	NS
CHE *U/liter*	2.4 ± 0.1	2.1 ± 0.18	NS
Mortality	$N = 9$	$N = 7$	NS

Patients were treated with different hemofilters: Group I, Multiflow60; Group II, FH66. Data are presented as mean ± SEM. Statistics were performed by the Student's *t*-test. Abbreviations are: GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transaminase; CHE, cholinesterase.

tional organ failure occurred. The cause of ARF was cardiac shock, sepsis, or trauma.

Patients were treated consecutively with CVVH using either (group I) hollow fiber polyacrylonitrile hemofilter (Multiflow60; Hospal, Nürnberg, Germany) or (group II) polyamide hollow fiber hemofilter (FH66; Gambro Co., Hechingen, Germany). In total, 16 patients were treated with Multiflow60 hemofilters, and 15 patients were treated with the FH66 hemofilter.

The mean age and liver specific parameters [glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and cholinesterase (CHE)] of patients in both groups did not differ (Table 1). The urea concentration and residual renal function measured by the amount of daily urine production were significantly lower in group II (Table 1).

Ultrafiltrate collection and treatment protocol

Vascular access was provided by double-lumen dialysis catheter of the jugular or femoral vein. Each hemofilter and blood lines were initially rinsed with 2 liters of normal saline solution, including IE heparin. Patients were connected directly to the extracorporal circuit. Blood flow was 60 to 80 ml/min for group I (machine type: BSM21; Hospal) and 100 to 120 ml/min for group II (machine type: ADM08; Fresenius Medical Care, Bad Homburg, Germany). Heparin is given by 500–1000 IE/hr. The first 50 ml of ultrafiltrate were removed in order to avoid contamination with rinsing solution. Thereafter, 100 ml of pure ultrafiltrate (without rinsing solution, without dialysate) were collected for analysis. Samples were cooled on ice, sterile filtered (0.2 μm), aliquoted under a laminar flow hood, and stored at −20°C until further use.

Culture techniques

Hepatoma-derived cell line HepG2 (ATCC: HB-8065) were routinely cultured in RPMI-growth medium (RPMI 1640, Biochrom F1215) containing 10% fetal calf serum, 1% glutamine, and 1% streptomycin/penicillin. Cells were weekly subcultured and seeded in 96- or 24-well plates (No. 655180; Greiner Labortechnik, Solingen, Germany) in a density of 100,000 cells per well or 500,000 cells per well. After 24 hours, growth medium was removed, and cells were incubated for 24 to 48 hours with ultrafiltrates of the two different hemofilters. All media had a low endotoxin level (≤0.2 EU/ml). The vitality of cells, as assessed by trypan blue exclusion test, was always greater than 95%.

Assays

LDH assay. Cytotoxicity of ultrafiltrates was assessed by the release of LDH into the medium. Measurements were performed using a commercially available assay (Roche Diagnostics GmbH., Mannheim, Germany) according to the instructions of the manufacturer.

Proliferation assay. Cellular proliferation was determined by bromodeoxyuridine (BrdU) incorporation using a commercially available assay (Roche Diagnostics GmbH., Mannheim, Germany). Following labeling with BrdU, incorporated BrdU was detected using a peroxidase conjugated anti-BrdU antibody. The substrate reaction was terminated by 1 M sulfuric acid, and absorbences were read at 450 nm. Medium controls were arbitrarily set at 100%.

Enzyme-linked immunosorbent assay (ELISA) of acute-phase proteins (transferrin, α1-acid glycoprotein). Acute-phase proteins were measured in cell-conditioned media after 48 hours of incubation with different ultrafiltrates. Immunoplates (NUNC, Wiesbaden, Germany) were coated with first antibody (mouse anti-human transferrin or α1-acid glycoprotein, Biogenesis, Berlin, Germany) overnight at 4°C, blocked with 100 μl of phosphate buffered saline (PBS)/0.05% Tween 20/0.25% bovine serum albumin (BSA), and incubated with 100 μl of antigen (standard of transferrin or α1-acid glycoprotein, Biogenesis, Berlin, Germany) or cell supernatant for one hour at room temperature. PBS/Tween-buffer was used for washing between each step. The ELISA sandwich was competed by horseradish peroxidase-conjugated antibody (transferrin or α1-acid glycoprotein, Biogenesis, Berlin, Germany). O-phenylendiamine dihydrochloride (OPD; Sigma-Aldrich Co., Deisenhofen, Germany) was used as substrate solution. The reaction was terminated with 4 M sulfuric acid, and the absorbences were measured in a Dynatech microplate reader at dual wavelength (490 and 630 nm). The results were calculated by nonlinear regression analysis.

Protein detection in cell culture assay. Cells were lyzed in 0.1 M NaOH by overnight incubation at room temperature. Protein content was measured with a slightly modified Bradford-Assay using bovine serum albumin for calibration.

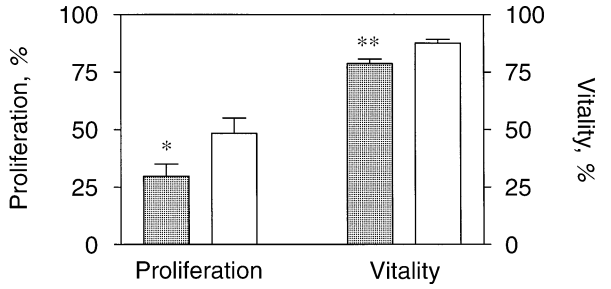

Fig. 1. Effects of ultrafiltrates on HepG2 cells. HepG2 cells were incubated with different ultrafiltrates for 24 hours [(■), group I, Multiflow60 ($N = 16$), or (□) group II: FH66 ($N = 15$)]. LDH release (vitality) and proliferation (BrdU incorporation) were determined as described in the experimental procedures. Data are presented as mean ± SEM. Statistical differences are analyzed by one-way analysis of variance. Vitality: **group I vs. group II, $P < 0.01$. Proliferation: *group I vs. group II, $P < 0.05$.

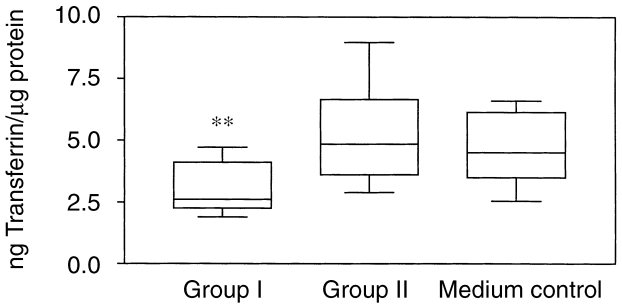

Fig. 2. Effect of ultrafiltrates on transferrin secretion by HepG2 cells. Cells were incubated with different ultrafiltrates for 48 hours [group I, Multiflow60 ($N = 16$), or group II, FH66 ($N = 15$)]. Secreted transferrin was measured by ELISA. Data (ng/µg total protein) are presented as box-plot diagrams, with the box encompassing the range of values from the 25% percentile (lower bar) to the 75% percentile (upper bar). The horizontal line within the box represents the median, and the lines above and below the box signify the maximum and minimum values. Statistical differences were analyzed by one-way analysis of variance. **Group I vs. group II: $P < 0.01$; *Group I vs. medium control: $P < 0.05$.

Statistics

All data were expressed as mean ± SEM. Statistical differences were tested by Student's *t*-test or by one-way analysis of variance.

RESULTS

Effects of ultrafiltrates on bromodeoxyuridine incorporation and lactate dehydrogenase release

Incubation of liver cells with ultrafiltrates significantly influenced their integrity and functionality compared with cells incubated with culture medium (Figs. 1–3).

Proliferation (group I 29.8 ± 5.2% vs. group II 48.4 ± 6.6%, $P < 0.05$; Fig. 1) and vitality (group I 78.7 ± 2.0% vs. group II 87.6 ± 1.7%, $P < 0.01$; Fig. 1) of HepG2 cells were significantly stronger inhibited by Multiflow60 ultrafiltrates (group I).

Effects of ultrafiltrates on acute-phase protein secretion

Secretion of the negative acute-phase protein transferrin in the supernatants of HepG2 cells was significantly reduced by Multiflow60 ultrafiltrates (group I) compared with medium control (group I: 3.1 ± 0.2 ng/µg protein vs. group II 5.1 ± 0.5 ng/µg protein, $P < 0.01$; group I vs. medium control 4.8 ± 0.5 ng/µg protein, $P < 0.05$; Fig. 2).

On the other hand, secretion of the positive acute-phase protein AGP (α1-acid glycoprotein) was significantly stimulated by Multiflow60 ultrafiltrates compared with medium control (group I 2.6 ± 0.1 ng/µg protein vs. group II 1.7 ± 0.1 ng/µg protein, $P < 0.001$; group I vs. medium control 1.7 ± 0.1 ng/µg protein, $P < 0.001$; Fig. 3).

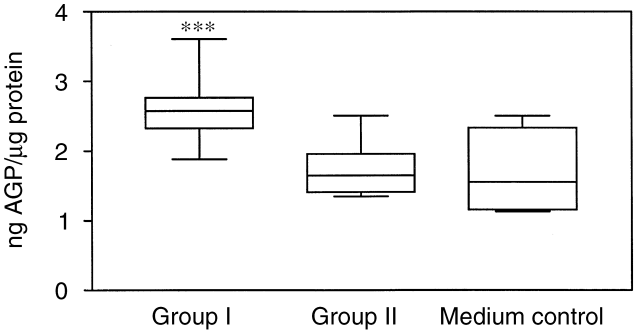

Fig. 3. Effect of ultrafiltrates on α1-acid glycoprotein (AGP) secretion by HepG2 cells. Cells were incubated with different ultrafiltrates for 48 hours [group I: Multiflow60 ($N = 16$) or group II: FH66 ($N = 15$)]. Secreted AGP was measured by ELISA. Data (ng/µg total protein) of three different incubation experiments are presented as box-plot diagrams, with the box encompassing the range of values from the 25th percentile (lower bar) to the 75th percentile (upper bar). The horizontal line within the box represents the median, and the lines above and below the box signify the maximum and minimum values. Statistical differences were analyzed by one-way analysis of variance. ***Group I vs. group II: $P < 0.001$; ***Group I vs. medium control: $P < 0.001$.

DISCUSSION

Continuous renal replacement therapies are known to have benefits in patients suffering from multi-organ failure [6]. The major advantages of CRRT compared with intermittent hemodialysis are improved cardiovascular stability, maintenance of cerebral perfusion, adequate metabolic control, and effective control of fluid balance, allowing adequate nutritional intake.

With respect to liver failure, literature is scarce. However, during bridging of acute liver failure patients to liver transplantation, the use of CRRT is recommended in the initial stage of kidney failure [7].

Studies demonstrating the effects of replacement therapies in a prospective randomized fashion were not available until now.

Our data demonstrate that ultrafiltrates eliminated

from patients with multiorgan failure activate hepatic acute-phase response and impair liver cell function.

Currently, we cannot describe the exact nature of all mediators removed by HF and the individual biological significance of their removal. A key finding from the incubation experiments was that ultrafiltrates significantly inhibited proliferation and vitality of HepG2 cells.

Besides their suppressing qualities, CVVH ultrafiltrates also demonstrated a stimulating effect on acute-phase protein secretion. The exact mechanism of ultrafiltrate-stimulated acute-phase protein response cannot be determined by our experiments. Involvement of cytokines seem to be unlikely, especially because Multiflow60 membrane in contrast to FH66 membrane is known to adsorb great amounts of cytokines during the first 15 to 30 minutes of HF [8–10]. Besides this, their filtration through membranes is limited by their molecular size, plasma protein binding, and receptor bindings [10]. However, C5a and possibly hepatocyte growth factor (HGF; 35 to 70 kDa), which has an additional antiproliferative effect on HepG2 cells, could be potential mediators of the acute-phase protein synthesis of HepG2 cells [11, 12]. Nevertheless, Multiflow60 membranes were found to adsorb large amounts of complement components, and the molecular weight of HGF is above the cut-off point of the synthetic membranes.

Our data also demonstrate different effects of ultrafiltrates on liver cells with regard to both treatment groups. One reason for this could be due to the different elimination behavior of the two membranes used. Some other reasons for the measured *in vitro* effects might be the differences in patients and treatment protocol. Diuresis and urea serum levels of patients differed in both treatment groups (Table 1). Furthermore, different types of machines had to be used for treatment (BSM21 vs. ADM08) requiring different blood flow through dialyzers in both groups. Moreover, ultrafiltrates were collected only at one time point (that is, filter change of the 24-hr treatment period). Thus, the data do not reflect all the effects of ultrafiltrate over 24 hours.

In conclusion, hepatoactive mediators can be ultrafiltrated by highly biocompatible synthetic membranes in critically ill patients with ARF. They are hepatotoxic and can stimulate the acute-phase response. Therefore, CRRT might be of potential benefit in order to prevent hepatic failure. This view is supported by recent investigations in which isovolemic HF improved specific organ failure in septic animals [13–15] and by positive clinical results in critically ill ARF patients with fulminant hepatic failure [3]. However, there are some primary topics that should be investigated in detail in the future. Differences between the ultrafiltrates of the two membranes (Multiflow60 vs. FH66) have to be elucidated. Detailed biochemical analyses of the putative mediators must be done to identify their natures. Clinical randomized studies should try to clarify the effects of CRRT on liver function.

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